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**Endocannabinoid activation of CB<sub>1</sub> receptors contributes to long-lasting reversal of neuropathic pain by repetitive spinal cord stimulation.**

Running title: **Endocannabinoids in spinal cord stimulation anti-hyperalgesia.**

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## **Abstract**

**Background:** Spinal cord stimulation (SCS) has been shown to be effective in the management of certain neuropathic pain conditions, however, the underlying mechanisms are incompletely understood. In this study, we investigated repetitive SCS in a rodent neuropathic pain model, revealing long-lasting and incremental attenuation of hyperalgesia and a mechanism of action involving endocannabinoids. **Method:** Animals were implanted with monopolar electrodes at the time of partial sciatic nerve injury. Dorsal columns at spinal segments T12/13 were stimulated 3 days later (early-SCS), and again at day 7 (late-SCS) using low frequency parameters. Hypersensitivity to cutaneous mechanical stimuli was assessed using von Frey filaments. Pharmacological agents, selected to identify endocannabinoid and opioid involvement, were administered intraperitoneally, 10 min before SCS. **Results:** Early-SCS caused partial reversal of mechanical hypersensitivity with corresponding changes in the biomarker of central sensitization, [phospho-Tyr<sup>1472</sup>]-GluN2B. The partial reversal of hyperalgesia by early-SCS was amplified by co-administration of LY 2183240, an inhibitor of endocannabinoid reuptake/breakdown. This amplification was inhibited by a CB<sub>1</sub>R antagonist, AM251, but not by a CB<sub>2</sub>R antagonist, AM630. Early-SCS-induced reversal of hyperalgesia was attenuated by naloxone, indicating a role for opioids. Late-SCS resulted in an incremental level of reversal of hyperalgesia, which was inhibited by AM251, but not by CB<sub>2</sub> or opioid receptor antagonists. **Conclusion:** The endocannabinoid system, and in particular the CB<sub>1</sub>R, plays a pivotal role in the long-lasting and incremental reversal of hyperalgesia induced by repetitive SCS in a neuropathic pain model.

**Significance (What does this study add?):**

Alternative parameters for repetitive spinal cord stimulation (SCS) at 25/10Hz elicit particularly long-lasting and incremental reversal of hyperalgesia in a neuropathic pain model through a mechanism involving endocannabinoids.

## **1. Introduction:**

Neuropathic pain, caused by direct damage to the somatosensory nervous system, is inadequately treated by currently available analgesic agents (Finnerup et al., 2015). Neuromodulation using epidural spinal cord stimulation (SCS) is considered clinically when other treatment modalities fail. SCS targeting spinal dorsal column fibre tracts has been described since 1967 (Molnar and Barolat, 2014; Shealy et al., 1967). Although SCS is an evidence-based and validated procedure for management of neuropathic pain in various clinical indications (Kumar et al., 2007; Slangen et al., 2014), the underlying mechanisms are still incompletely understood.

The rationale behind SCS was initially based on the Gate Control Theory of Pain (Melzack and Wall, 1965), whereby SCS was anticipated to result in antidromic activation of large myelinated A $\beta$  fibres and thereby cause suppression of nociceptive processing in superficial dorsal horn. Current evidence indicates greater complexity, involving spinal neuronal microcircuits, microglia and astrocytes, as well as activation of bulbospinal modulatory pathways that may reflect a role of opioids (Sato et al., 2014; Smits et al., 2013; Zhang et al., 2015). SCS (at various frequencies and intensities) can reverse neuropathic reflex hypersensitivity (Maeda et al., 2008; Pluijms et al., 2013; Yang et al., 2011). Repetition of SCS can lead to an incremental increase in reversal of hyperalgesia (Shechter et al., 2013), suggesting that initial SCS intervention might be able to elicit a priming (pre-conditioning) effect that could enhance the efficacy of subsequent treatments).

Endocannabinoids are bioactive lipids that are rapidly produced following cell activation. In the nervous system, both neurons and glia may contribute to their production in response to

Ca<sup>2+</sup>-mobilizing stimuli such as activation of Gq-coupled receptors, strong depolarization or spiking activity caused by damaging stimuli (Maccarrone et al., 2014). SCS might therefore increase endocannabinoid production to achieve analgesia in the partial sciatic nerve ligation (pSNL)-induced pain state. Endocannabinoids seem overall to exert analgesic (Ulugol, 2014) or neuroprotective influences (Arevalo-Martin et al., 2012). Endocannabinoids act mainly on CB<sub>1</sub> receptors (CB<sub>1</sub>Rs), highly expressed in both neuron and astrocytes, and CB<sub>2</sub> receptors (CB<sub>2</sub>Rs), limited to microglia and macrophages (Zurolo et al., 2010). CB<sub>1</sub>R agonists can produce reversal of hyperalgesia in models of neuropathic pain, at doses lacking psychotropic effects (Vera et al., 2013). CB<sub>2</sub>Rs have also been targeted for suppress of hyperalgesia (Guindon and Hohmann, 2008), partly because their expression in sensory pathways increases following nerve injury (Beltramo et al., 2006).

This study using a neuropathic pain model aimed firstly to investigate the effects of low frequency SCS early after nerve injury as well the effects of further, repeated, SCS (late SCS). Secondly it aimed to directly address, for the first time, the molecular basis for the long-lasting or incremental reversal of hyperalgesia induced by repetitive SCS. We hypothesized that early SCS-induced reversal of hyperalgesia might outlast the stimulation period and that further amplification of this effect might be achieved by second (late) SCS-treatment. The roles of endocannabinoids contributing to the development and/or maintenance of SCS-induced reversal of hyperalgesia and the receptor subtypes involved were also explored.

## **2. Materials and methods:**

### *Animals and experimental design:*

Adult male Sprague-Dawley rats weighing 200-250g were given food and water ad libitum and housed 1-2 per cage, at 21°C, 50% humidity, on a 12h light:12h dark schedule. The procedure was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), the University of Hong Kong. The rats were allocated to 4 main groups: 1) naïve; 2) partial sciatic nerve ligation (pSNL); 3) pSNL with spinal cord stimulation (pSNL+ SCS), 4) pSNL+ SCS with administration of drugs, (n=4-5 in each case). Additional minor groups for control experiments included naïve + SCS, pSNL + sham SCS (no current passed) and pSNL + single occasion SCS. The drugs investigated included the blocker of endocannabinoid reuptake and hydrolysis by fatty acid amide hydrolase (FAAH), LY 218340, and selective antagonists at CB<sub>1</sub>Rs (AM251), CB<sub>2</sub>Rs (AM630) or opioid Rs (naloxone), alone or in appropriate combinations, (n=3-5 in each case).

### *Surgery:*

The partial sciatic nerve ligation (pSNL) neuropathic pain model is a well characterised, previously described model (Tai et al., 2013). Briefly, rats were anaesthetised by inhalation of an isoflurane/O<sub>2</sub> mixture, 4-5% for induction and 1.5-3% for maintenance. Animals were carefully monitored during surgery until recovery from anaesthesia. Following hair removal and sterilization of the area with ethanol and betadine, a small incision was made below the pelvis and the right biceps femoris and the gluteus superficialis were carefully separated to expose the sciatic nerve. Upon isolation of the nerve, a ligation around one third to one half the diameter of the sciatic nerve was made to the nerve with 7-0 silicon-treated silk suture. The wound was then closed with skin sutures (4-0 Nylon). pSNL rats consistently developed

ipsilateral mechanical allodynia, which consistently peaked by day 3 post-surgery and was maintained at a similar level until day 10, the period in which SCS was delivered.

Electrodes were implanted epidurally immediately after sciatic nerve ligation. In contrast to previous studies where a second surgery was carried out for electrode implantation (Smits et al., 2006), we carried out the ligation and implantation together to simplify our protocol as the time needed for development of mechanical hypersensitivity and recovery from electrode implantation were 3 days in both cases. Hair on the back was shaved and the area sterilised with alcohol and betadine. The spinal column was exposed by a midline lumbar incision followed by laminectomy at T12-T13. The dura was kept intact. Following procedures described in detail previously (Li et al., 2006; Smits et al., 2006), a rectangular platinum-iridium monopolar electrode (3×0.5×0.1 mm; cathode) was introduced into the dorsal epidural space at T13. As this site is a considerable distance from the input location of afferents from the plantar surface of the hind paw (around L5), effects on sensory responses elicited there are likely to involve activation of neuronal tracts, rather than a direct influence on the central endings of afferents innervating the hind paws. A round platinum electrode (diameter: 6 mm; anode) was placed subcutaneously in the chest wall. A dissecting microscope was used to enable accurate placement and minimise damage. The wires from both electrodes were tunnelled under the skin to the neck to make contact with a pair of connectors. The connectors exited the skin on back of the neck to connect with wires from the external pulse stimulator. The wound was then closed with sutures (4-0 Nylon). Behavioural assessment was carried out 3 days later.

*Mechanical threshold measurement:*



Among the most problematic characteristics of neuropathic pain in the clinic is hypersensitivity to mechanical sensory stimuli resulting in mechanical allodynia. We focused here on assessing this on both ipsilateral and contralateral hind paws of rats using an electronic von Frey Aesthesiometer (IITC, USA). Animals were placed on a raised mesh grid and covered with a clear plexiglass box to contain the animal in a red-light/dark environment. They were given 10-15 minutes in their new test environment to allow them to become habituated before testing. Von Frey filament testing allows determination of the threshold force for withdrawal of the ipsilateral paw compared to the contralateral paw. As described previously (Moss et al., 2002), filaments in order of increasing force were applied to the middle of the plantar surface of each paw (avoiding the footpads) from below for 4-6 sec or until the filament bent. After the first positive withdrawal response to a particular filament, the von Frey test was repeated three times, using this filament, to ensure reproducibility. The threshold reading (in grams) was recorded.

#### *Electrical stimulation:*

Animals were allowed to recover for 3 days after pSNL surgery and the implantation of spinal cord electrodes. We then applied the first spinal cord stimulation (SCS1; early-SCS) using mild, deliberately suboptimal, parameters that would allow detection of any incremental change associated with subsequent stimulation. SCS1; early-SCS was applied for 10 min with a frequency of 25 Hz, a pulse width of 0.05 ms and a stimulation intensity of approximately 60% of motor twitch threshold. In reports providing evidence for incremental reversal of hyperalgesia following repetitive SCS, the effect is most obvious at submaximal stimulation intensities and at relatively low frequencies of 4-50 Hz (Maeda et al., 2008; Shechter et al., 2013; Yang et al., 2011). We therefore used a modest stimulation intensity

and selected 25 Hz as the lowest frequency that produced approximately 50% reversal of pSNL mechanical hypersensitivity in our pilot experiments. As preliminary experiments indicated that the anti-hyperalgesic effect appeared to be largely maintained for up to 4 days, the second period of SCS (SCS2; late-SCS) was not applied until that time. SCS2; late-SCS parameters were the same as for SCS1, except that we used a frequency of 10 Hz (which when tested in preliminary experiments, as the first stimulation, produced only a minimal, 15-20% reversal of mechanical hypersensitivity). These parameters were well-suited to one of the key aims, which was to explore the processes by which an initial SCS might modify the efficacy of a subsequent SCS event. The withdrawal responses to mechanical stimuli were assessed 30 minutes after each stimulation. Excluding animals with failed surgery or electrode placement, or broken connectors, the success rate of animals developing mechanical hypersensitivity and responding to SCS after implantation of the electrodes was 100%.

#### *Drug administration:*

The blocker of endocannabinoid reuptake and FAAH, LY 2183240 ((Dickason-Chesterfield et al., 2006); 1mg/kg), the selective CB<sub>1</sub>R antagonist, AM251 ((Lan et al., 1999); 1.5 mg/kg), the selective CB<sub>2</sub> antagonist, AM630 ((Ross et al., 1999); 3 mg/kg), and the broad spectrum opioid receptor antagonist, naloxone (10 mg/kg), were made up in a vehicle of 2% dimethylformamide (aqueous) and injected intraperitoneally (alone or in combination) 10 min before spinal cord stimulation. The vehicle alone had no discernible effect. Compounds were obtained from MedChem Express, USA; Tocris, UK; or Sigma-Aldrich, UK.

#### *Immunoblotting:*

Dorsal spinal cords from lumbar segments (L3-5) were harvested 10 days after partial sciatic nerve ligation, or 3 days after SCS1, or 3 days after SCS2 and lysed in Laemmli buffer, (n=3 in each case). Proteins were separated by SDS-PAGE on 8% Tris-acetate gels before transfer to polyvinylidenedifluoride membrane. Protein loading on gel lanes was carefully balanced by preliminary experiments, firstly using Coomassie Blue staining and then immunoblot for  $\alpha$ -tubulin to adjust sample loading volumes to those that would provide equalised protein content. After blocking with 5% BSA, membranes were incubated overnight at 4°C with mouse monoclonal anti- $\alpha$ -tubulin (1: 1000, Sigma-Aldrich, UK) for load balancing or with rabbit anti-[phospho-Tyr<sup>1472</sup>]-GluN2B (1:1000, Millipore, Germany) or rabbit anti-pan GluN2B (1:1000, Millipore, Germany), followed by incubation with peroxidase-conjugated secondary antibodies; goat anti-mouse or goat anti-rabbit IgG (1:10,000, Cell Signaling Technology, USA) for 1h at room temperature. Staining was detected by chemiluminescence using X-ray film. Films were scanned for grey-scale densitometric analysis using Image-J (freely available from NIH, USA).

#### *Statistical analysis:*

All experiments were repeated at least three times. All numerical data were presented as means  $\pm$  standard error. Data were analyzed using One-Way or Two-Way Analysis of Variance with Tukey's post-hoc test. When p values were less than 0.05, differences were considered significant. All analyses were carried out using GraphPad Prism 6.

### 3. Results:

#### *Reversal of mechanical allodynia by SCS:*

To quantify the anti-hyperalgesic effect of SCS, the paw withdrawal threshold (PWT) of pSNL rats before and after first SCS (SCS1; early-SCS) and second SCS (SCS2; late-SCS) was assessed. Ipsilateral PWT was decreased by pSNL before SCS1 on post-operative day 3 from  $54.6 \pm 0.7$  g to  $20.2 \pm 1.0$  g ( $p < 0.0001$ ), but this decreased ipsilateral PWT was significantly elevated by SCS1 (at 25 Hz) to  $36.9 \pm 1.7$  g ( $p = 0.0005$ ); around 49% reversal of hypersensitivity (Fig. 1). We also assessed how long the anti-hyperalgesic effect elicited by SCS1 would last, so allowed a gap of 4 days before re-testing. It was shown that the elevation of ipsilateral PWT induced by SCS1 was only slightly diminished to around 40% reversal of hypersensitivity by this time point (Fig. 1). SCS2 (at 10 Hz, which in the absence of pre-conditioning by SCS1 caused only a minimal 15-20% attenuation of hypersensitivity) was given on day 4 after SCS1. This significantly raised the PWT from  $34.0 \pm 1.7$  g to  $49.3 \pm 1.1$  g ( $p = 0.0022$ ); now representing a marked 85% reversal of hypersensitivity (Fig. 1). Re-testing 3 days later, just before animals were culled, showed that ipsilateral PWT values had declined to  $39.0 \pm 4.0$  g; around 55% reversal of hypersensitivity (Fig. 1). A group of animals subjected to pSNL and SCS1 (early-SCS) only showed a gradual progressive decline in the anti-hyperalgesic influence of SCS1 such that PWT values had almost completely returned to ipsilateral pSNL scores by 7 days later. Sham SCS animals, in which pSNL and electrode implantation surgery was carried out but no current passed, showed no discernible differences from pSNL alone animals either ipsilateral or contralateral to nerve injury (Fig. 1). Further control experiments showed no detectable effect of SCS1 stimulation on PWT values in naive animals (matching the consistent lack of effect seen on the contralateral, uninjured side in pSNL animals ( $n=4$ , data not shown)). Our SCS protocol thus resulted in a relatively long lasting reversal of mechanical allodynia due to peripheral nerve ligation and in incremental

enhancement of anti-hyperalgesic effect upon repetitive stimulation. No discernible changes were observed in contralateral PWT due to either pSNL or SCS.

#### *Inhibition of spinal NMDA receptor phosphorylation by SCS:*

NMDA-type receptors for the excitatory transmitter glutamate play a key role in the central sensitization of spinal cord neurons that underpins chronic hypersensitive pain states (Millan, 1999; Woolf and Thompson, 1991). To monitor a biomarker of hypersensitivity in the dorsal spinal cord after peripheral nerve ligation with or without SCS, we examined the activation of NMDARs, reflected by phosphorylation of the GluN2B subunit at Tyr-1472 (Nakazawa et al., 2001). The densitometric ratio of the immunoblot bands (running at 180 kDa) for [phospho-Tyr<sup>1472</sup>]-GluN2B to pan-GluN2B in ipsilateral dorsal spinal cord was significantly increased following pSNL ( $p < 0.01$ ; Fig. 2). Corresponding values for tissue collected on day 4 after SCS1 or 2 were significantly decreased, compared with that without electrical stimulation ( $p < 0.05$ ; Fig. 2). Although it is possible that NMDAR phosphorylation was even more marked immediately after SCS, tissue was collected at these time points (where we had established that SCS reversal of hyperalgesia was largely maintained) in order to minimize animal usage.

#### *Endocannabinoid-mediated, CB<sub>1</sub>R-dependent amplification of SCS1-induced reversal of hyperalgesia:*

To ascertain whether endocannabinoids might have a role in, or influence on, SCS1-induced reversal of hyperalgesia, the inhibitor of anandamide re-uptake and FAAH, LY 2183240, was injected (i.p.) 10 min before SCS1. LY 2183240 is expected to increase the endocannabinoid concentration by delaying degradation (Dickason-Chesterfield et al., 2006; Ulugol, 2014). As

contralateral PWT values were unaffected by pSNL or SCS (Fig 1) or by any of the pharmacological agents used in the study, further data assessing neurochemical mediators involved in SCS reversal of hyperalgesia were expressed as ipsilateral:contralateral PWT ratio, for simplicity. The PWT ratio of ipsilateral to contralateral sides was significantly reduced due to pSNL ( $p<0.01$ ) and this change was partially reversed by SCS1 ( $p<0.01$ ). In the presence of 1mg/kg of LY 2183240, the SCS1-induced reversal of hypersensitivity was significantly amplified ( $p<0.01$ ), compared with SCS1 alone (Fig. 3). To identify the downstream mediator of endocannabinoid action, AM251 or AM630, CB<sub>1</sub>R and CB<sub>2</sub>R antagonists respectively, were injected together with LY 2183240. The increased PWT ratio due to SCS1 in the presence of LY 2183240 was fully and significantly blocked by AM251, a CB<sub>1</sub>R antagonist ( $p<0.01$ ), but not significantly altered by AM630, a CB<sub>2</sub>R antagonist. Neither LY 2183240 nor AM 251 alone had any discernible effect on pre-SCS1 PWT values and AM 251 alone caused no significant alteration in the effect of SCS1. None of the interventions showed any detectable effect on contralateral PWT values. These findings indicate that inhibiting endocannabinoid clearance facilitated SCS1-induced reversal of hyperalgesia, but alone (in the absence of SCS) caused no detectable change in reflex nociceptive behaviour, showing that the endocannabinoid action related specifically to SCS.

*Direct CB<sub>1</sub>R-dependence of SCS2-induced reversal of hyperalgesia:*

To evaluate whether endocannabinoids acting on CB<sub>1</sub> or CB<sub>2</sub>Rs contribute directly to the SCS2-induced anti-hyperalgesic effect, AM251 or AM630 were injected 10 min before SCS2. The ipsilateral:contralateral PWT ratio 30 min after SCS2 was significantly reduced by AM251 ( $p<0.01$ , compared with SCS2 alone), representing  $49.1 \pm 2.2$  % reversal of the anti-hyperalgesic effect of SCS2. AM630 had no discernible effect on SCS2-induced responses

(Fig. 4). None of the interventions showed any detectable effect on contralateral PWT values. These findings indicated that the increased anti-hyperalgesic effect induced by SCS2, following priming by SCS1, presumably involved the release of endocannabinoids, which exert their effects through CB<sub>1</sub>Rs. While the anti-hyperalgesic effect of SCS2 was directly sensitive to AM 251, the effect of SCS1 required amplification with LY 2183240 before any effect of AM 251 was detected. This is consistent with the possibility that conditioning by the repeated SCS stimulation may induce greater involvement of endocannabinoids acting on CB<sub>1</sub>Rs.

*Opioid receptor involvement in SCS1-, but not SCS2-induced reversal of hyperalgesia:*

A previous report indicated the involvement of opioids in SCS-induced reversal of mechanical hypersensitivity following peripheral nerve injury, although stimulation was carried out at much higher intensity and duration (Sato et al., 2013). We therefore investigated whether opioids might be involved in SCS-induced reversal of hyperalgesia under our conditions, with the secondary aim of establishing that any role of endocannabinoids was distinct and independent of opioid effects. The opioid receptor antagonist naloxone was therefore injected (i.p.) at a dose of 10 mg/kg, 10 min before SCS1 or SCS2. Naloxone is a broad spectrum opioid receptor antagonist with some selectivity for  $\mu$ -type receptors. It was found that naloxone significantly decreased the ipsilateral:contralateral PWT ratio after SCS1 when compared with SCS1 alone ( $p < 0.01$ ), representing  $64.7 \pm 3.0$  % inhibition of the anti-hyperalgesic effect of SCS1. Naloxone had no discernible effect on SCS2-induced reversal of hyperalgesia (Fig. 5). None of the interventions showed any detectable effect on contralateral PWT values. These findings

showed a role of opioids in the acute anti-hyperalgesic effect of SCS1 under our conditions, a completely different profile from that shown by endocannabinoids.

#### **4. Discussion:**

Although the SCS technique has been used for pain relief for several decades, the underlying neurobiological mechanisms remain incompletely understood. We show that SCS can alleviate mechanical allodynia caused by peripheral nerve ligation, in accordance with previous reports (Li et al., 2006; Maeda et al., 2008; Shechter et al., 2013; Smits et al., 2006; Truin et al., 2011b; Yang et al., 2011), but further demonstrate long-lasting and incremental anti-hyperalgesic effects of repetitive SCS and address aspects of the underlying mechanisms.

The anti-hyperalgesic efficacy of SCS is dependent on stimulation parameters, such as frequency. A wide range of frequencies from 4 Hz to 10 kHz has been experimentally evaluated (Sato et al., 2014; Shechter et al., 2013). SCS at frequencies of 10-50 Hz can provide relief from cancer pain (Shealy et al., 1967). Although both high frequency and conventional frequency (50 Hz) can attenuate hypersensitivity in neuropathic pain states (Yang et al., 2011), low frequency (4 Hz) rather than high frequency (100 Hz) preferentially attenuated mechanical allodynia in a peripheral neuropathic pain model (Maeda et al., 2008). We focused here on low frequency stimulation to ensure that we were well within the frequency range through which most spinal cord neurons can maintain firing of action potentials, to optimise the possibility of observing incremental effects upon repetitive SCS. Our results show that low intensity monopolar SCS at 25 Hz can alleviate the mechanical allodynia caused by peripheral nerve injury. Unexpectedly, our protocol resulted in partial but prolonged maintenance of SCS-induced reversal of hyperalgesia over several days. This has



not been widely described previously but is consistent with earlier reports where the effect of repetitive daily SCS had not fully declined before the next day's session (Maeda et al., 2008; Shechter et al., 2013; Yang et al., 2011). The molecular basis is unknown, but is of interest as a potential target for enhancing the efficacy and maintenance of SCS-induced relief of nociceptive hypersensitivity.

Timing and repetition are further important parameters. While significant reversal of hyperalgesia was generated here by SCS1 (early-SCS), a much greater alleviation of hypersensitivity was achieved by SCS2 (late-SCS), delivered at a lower frequency of 10 Hz (which itself produced minimal analgesia in the absence of pre-conditioning by SCS1). This incremental efficacy upon repetitive SCS is consistent with, but more marked than, observations in previous reports (Maeda et al., 2008; Shechter et al., 2013; Yang et al., 2011). In our previous work, which used 50 Hz monopolar stimulation over a longer 30 min period (Smits et al., 2006), the acute anti-hyperalgesic effect largely reversed within 1 hour. However, when SCS was applied early after pSNL, the effect was longer lasting than in established (16 day) pSNL animals (Truin et al., 2011a). The milder submaximal stimulus parameters used here in conjunction with the relatively earlier SCS intervention, 3 days following nerve injury may make it easier to evoke long-lasting and incremental SCS-induced reversal of hyperalgesia.

The behavioural measurements were strongly corroborated by Western blot data showing that levels of [phospho-Tyr<sup>1472</sup>]-GluN2B, a biomarker of central sensitization (Nakazawa et al., 2001), in ipsilateral dorsal spinal cord were increased following pSNL and that this effect was reversed by SCS1 or SCS2. The increased [phospho-Tyr<sup>1472</sup>]-GluN2B to pan-GluN2B

ratio induced by pSNL was recorded at >3 days when the mechanical hypersensitivity had fully developed and stimulus-induced protein phosphorylation had become established. SCS1 and SCS2 caused partial reversal of this increase in tissue taken 4 days subsequently; an adequate time for SCS to re-set the NMDAR phosphorylation state. Although SCS was only delivered for brief periods, its behavioural impact was largely maintained over several days and this was reflected by a maintained reduction in NMDAR phosphorylation. This indicates that SCS may reduce the central sensitization caused by pSNL partly by inhibiting the activation of spinal NMDARs. We showed previously that low doses of the NMDAR antagonist ketamine (that do not show an explicit anti-hyperalgesic effect) can increase the efficacy of SCS; supporting the idea that the activation status of the NMDAR may be important in SCS reversal of hyperalgesia (Truin et al., 2011a). The present study reports a highly efficacious stimulation scheme for reversal of neuropathic hypersensitivity, validated at both behavioural and biochemical levels.

We also demonstrated that endocannabinoids and cannabinoid receptors contribute to SCS-induced reversal of hyperalgesia in the pSNL model. Using LY 2183240 to increase endocannabinoid concentration (by inhibiting anandamide uptake and suppressing breakdown through FAAH), we observed amplification of the anti-hyperalgesic effect of SCS1. Endocannabinoids act predominantly through CB<sub>1</sub> and CB<sub>2</sub> receptors, which are mainly expressed in the nervous system and immune system, respectively. We showed here that the amplification of SCS1-induced reversal of hyperalgesia was CB<sub>1</sub>R-dependent and further that the effect of SCS2 directly relies on endocannabinoid action via CB<sub>1</sub>Rs. While this influence may reflect an indirect influence of endocannabinoids on the excitability of NMDAR-expressing neurons, there are intriguing reports of the CB<sub>1</sub>R directly associating with NMDAR to negatively regulate the CB<sub>1</sub>R:NMDAR, which is required for endocannabinoid-

induced reversal of hyperalgesia (Sanchez-Blazquez et al., 2014; Sanchez-Blazquez et al., 2013). CB<sub>1</sub>R activation is also known to inhibit NMDAR-induced Ca<sup>2+</sup> elevation in cultured hippocampal slices (Hampson et al., 2011). We did not attempt to assess facilitatory effects of cannabinoids in the NMDAR phosphorylation paradigm, because of the more limited dynamic range of this assay. While it is possible that endocannabinoid facilitation of the anti-hyperalgesic efficacy of SCS may relate to altered NMDAR activation, endocannabinoids are known to have multiple sites of pre- and post-synaptic action (Pertwee, 2001; Ulugol, 2014; Walker and Huang, 2002), so additional processes are likely to contribute. CB<sub>1</sub>Rs are also expressed in astrocytes (Stella, 2010; Zurolo et al., 2010) and models of neuropathic pain are reported to result in activation of spinal cord microglia (with activation of astrocytes in some but not other studies) (Ikeda et al., 2012; Matsuo et al., 2014; Miller et al., 2013). The CB<sub>1</sub>Rs involved here in SCS-induced reversal of hyperalgesia seem likely to be located neuronally, although an astrocytic location cannot be excluded. CB<sub>1</sub>Rs are abundantly expressed in both excitatory and inhibitory interneurons of the superficial dorsal horn in spinal cord; at locations consistent with participation in the effects observed here (Salio et al., 2002; Yang et al., 2016). CB<sub>2</sub>R expression is induced in spinal cord in neuropathic pain models (Zhang et al., 2003) and may have potential value as a therapeutic target (Brownjohn and Ashton, 2012; Ibrahim et al., 2005; Rahn et al., 2008; Whiteside et al., 2005). Nonetheless, our evidence points to a key role for CB<sub>1</sub>Rs, rather than CB<sub>2</sub>Rs, in SCS-induced relief of neuropathic hyperexcitability. There is evidence that microglia are activated in neuropathic pain models (Ikeda et al., 2012; Matsuo et al., 2014; Miller et al., 2013), and various formats of electrical stimulation, including SCS, can inhibit this (Hahm et al., 2015; Hsu et al., 2015; Matsuo et al., 2014; Sato et al., 2014). However, the fact that microglial CBRs are CB<sub>2</sub> rather than CB<sub>1</sub> may mean that microglia are not substantially involved in the CB<sub>1</sub>R-dependent anti-hyperalgesic effect observed here following repetitive SCS.

Opioid receptor activation was reported to play a role in the reversal of hyperalgesia in a nerve injury model induced by intense and prolonged SCS in a frequency-dependent manner (Sato et al., 2013). We therefore evaluated the role of opioid receptors in the effects of repetitive SCS in present study. Opioid receptor inhibition by naloxone abolished the reversal of hyperalgesia induced by SCS1 (early SCS), but not the incrementally greater effect caused by SCS2 (late SCS). This indicates that in contrast to CB<sub>1</sub> receptors, opioid receptors participate in SCS1 but not SCS2-induced reversal of hyperalgesia. While CB<sub>1</sub>-opioid receptor heterodimerisation has been reported in the cortex of neuropathic animals (Bushlin et al., 2012), our evidence for distinct temporal involvement suggests that any such complex is not substantially involved here. Our mild SCS parameters appear to involve opioid-dependent reversal of hyperalgesia at initial intervention in established neuropathic pain, as seen with more intense and prolonged stimuli (Sato et al., 2013), but subsequently involve endocannabinoids instead. Different parameters are likely to underlie any mechanistic differences.

To conclude, we have shown that refinement of SCS parameters can markedly enhance the amplitude and duration of anti-hyperalgesic effects in a neuropathic pain model, and that this involves the endocannabinoid system. Our observations accord closely with a very recent report showing that SCS activation of A $\beta$ -fibres in a mouse neuropathic pain model caused CB<sub>1</sub>-dependent depression of C-fibre responses in superficial dorsal horn neurons (Yang et al., 2016). The present study is the first to reveal that endocannabinoid action at CB<sub>1</sub> receptors is specifically involved in the incremental and long lasting reversal of hyperalgesia induced by repetitive SCS stimulation. Using these SCS parameters, opioids appear to contribute to the reversal of hyperalgesia elicited by initial (but not subsequent) rounds of SCS. In contrast, endocannabinoids acting through CB<sub>1</sub> but not CB<sub>2</sub> receptors can amplify the

effects of early SCS and participate directly in the long-lasting and incremental relief of neuropathic hypersensitivity elicited by repetitive SCS.

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**Author contributions:**

Dr. Liting Sun carried out the experimental work including electrode implantation, spinal cord stimulation, pain-related behavioural testing and Western blots, and drafted the manuscript. Miss Lydia Tai helped in the partial sciatic nerve ligation. Dr. Qiu Qiu helped to prepare the electrical stimulation devices and performed the experiments. Prof. Sue Fleetwood-Walker and Dr. Rory Mitchell provided pharmacological agents, helped in the planning of experiments and reviewed the manuscript. Prof. Elbert Joosten supervised the progress and reviewed the manuscript. Dr. Chi Wai Cheung, as the corresponding author, and principal investigator, initially developed this study, supervised the progress, and reviewed the manuscript. All of the authors have discussed the results and commented on the manuscript.

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**Figure 1: Reversal of neuropathic hypersensitivity by SCS:** The graphs show mean  $\pm$  SEM values for mechanical paw withdrawal thresholds (PWT) in both ipsilateral and contralateral hind paws in nerve injured (pSNL) rats with or without spinal cord stimulation (SCS). Animals were implanted with electrodes at the same time as nerve injury, then the first electrical stimulation was carried out in the test group 3 days later, when hypersensitivity was fully developed. The second electrical stimulation was performed after a further 4 days when SCS1 (early-SCS)-induced reversal of hypersensitivity had only slightly declined. The animals were culled on day 10 after injury. A control group that received pSNL and SCS1 only, showed a progressive decline in the anti-hyperalgesic influence of SCS1, which had essentially disappeared by day 7 after SCS1. pSNL plus sham SCS controls (electrode implantation but no passage of current) showed no discernible differences from pSNL alone. The ipsilateral PWT following SCS1 (early-SCS) and subsequent SCS2 (late-SCS) was significantly increased (\*\*), comparing to that in animals without SCS. Moreover, it was also significantly increased after SCS1/2 (++), compared to that before each SCS session. \*\* $p < 0.01$  by Two-Way ANOVA with Tukey's post-hoc test. ++  $p < 0.01$  by Student's t test. No discernible changes were observed in the contralateral limb under any of the conditions tested (n=5 in each case).

**Figure 2: Nerve injury-induces an increase in dorsal spinal cord [phospho-Tyr<sup>1472</sup>]-GluN2B immunoreactivity that is attenuated by SCS:** Prominent immunoblot bands for [phospho-Tyr<sup>1472</sup>]-GluN2B and pan-GluN2B were seen at the expected running position of approximately 180 kDa, as indicated by the molecular weight marker. The bar charts below show the mean densitometric ratio for [phospho-Tyr<sup>1472</sup>]-GluN2B:pan-GluN2B in each case. pSNL nerve injury significantly increased levels of the active form of the pain-associated biomarker [phospho-Tyr<sup>1472</sup>]-GluN2B compared to pan-GluN2B in the dorsal spinal cord ipsilateral to partial sciatic nerve ligation. Both SCS1 (early-SCS; shown in (a) and SCS2 (late-SCS; shown in (b) significantly reduced the pSNL-induced increase in [phospho-Tyr<sup>1472</sup>]-GluN2B:pan-GluN2B ratio. \*p<0.05 and \*\*p<0.01 by One-Way ANOVA with Tukey's post-hoc test; n=4 in each case).

***Figure 3: Amplification of SCS-induced reversal of hyperalgesia in the pSNL neuropathic pain model by an inhibitor of endocannabinoid uptake/breakdown is CB<sub>1</sub>R-mediated:***

Mechanical paw withdrawal thresholds (von Frey filaments) were measured in the pSNL model of neuropathic pain and expressed as the ipsilateral:contralateral ratio, where low ratios after pSNL reflect the ipsilateral reduction in withdrawal threshold. Animals were implanted with electrodes at the same time as nerve injury, then electrical stimulation was carried out in the test group 3 days later, when hypersensitivity was fully developed. The CB<sub>1</sub>R antagonist, AM251 at 1.5 mg/kg caused no significant alteration in the anti-hyperalgesic effect of SCS1. However, the inhibitor of endocannabinoid re-uptake and breakdown by FAAH, LY 2183240, significantly amplified the analgesic effect of SCS1 (early-SCS) and this was significantly reversed by the CB<sub>1</sub>R antagonist, AM251, while neither had any effect alone in the absence of SCS. The CB<sub>2</sub>R antagonist AM630 had no significant effect on the amplification of the SCS1 effect caused by LY 2183240. \*\*p<0.01 compared to basal naïve values, ++ p<0.01 compared to Pre-SCS, ψψ p<0.01 compared to Post-SCS1, and ##p<0.01 compared to SCS1+L2183240 by One-Way ANOVA with Tukey's post-hoc test. No discernible changes were observed in PWT values for the contralateral limb (n=3-5 in each case).



**Figure 4: The anti-hyperalgesic effect of a second period of SCS in the pSNL neuropathic pain model is partly CB<sub>1</sub>R-mediated:** Mechanical paw withdrawal thresholds (von Frey filaments) were measured in the pSNL model of neuropathic pain and expressed as the ipsilateral:contralateral ratio, where low ratios after pSNL reflect the ipsilateral reduction in withdrawal threshold. Animals were implanted with electrodes at the same time as nerve injury, then the first electrical stimulation was carried out in the test group 3 days later, while the second electrical stimulation was performed after a further 4 days. The SCS2 (late-SCS)-induced increase in the ipsilateral:contralateral PWT ratio was attenuated by the CB<sub>1</sub>R antagonist, AM251, which was administered 10 min before SCS2. The CB<sub>2</sub>R antagonist, AM630 had no effect. \*\*p<0.01 compared to basal naïve values,  $\psi\psi$ p<0.01 compared to Pre-SCS, and ###p<0.01 compared to Post-SCS2 by One-Way ANOVA with Tukey's post-hoc test. No discernible changes were observed in the contralateral limb (n=3-5 in each case).

***Figure 5: Opioid receptors contribute to the anti-hyperalgesic effect of SCS1, but not SCS2:***

Mechanical paw withdrawal thresholds (von Frey filaments) were measured in the pSNL model of neuropathic pain and expressed as the ipsilateral:contralateral ratio, where low ratios after pSNL reflect the ipsilateral reduction in withdrawal threshold (\*\* $p < 0.01$  compared to basal naïve values). Animals were implanted with electrodes at the same time as nerve injury, then the first electrical stimulation was carried out in the test group 3 days later, while the second electrical stimulation was performed after a further 4 days. The SCS1 (early-SCS)-mediated increase in the ipsilateral:contralateral PWT ratio (++ $p < 0.01$  compared to pre-SCS) was significantly reduced by the opioid receptor antagonist, naloxone, which was administered 10 min before SCS1. †† $p < 0.01$  compared to Post-SCS1 by One-Way ANOVA with Tukey's post-hoc test. Ipsilateral:contralateral PWT ratios following SCS2 were also significantly greater than pre-SCS (ψψ $p < 0.01$ ), but naloxone caused no alteration in the anti-hyperalgesic effect of SCS2 (late-SCS). No discernible changes were observed in PWT values for the contralateral limb ( $n=4-5$  in each case).

Fig. 1

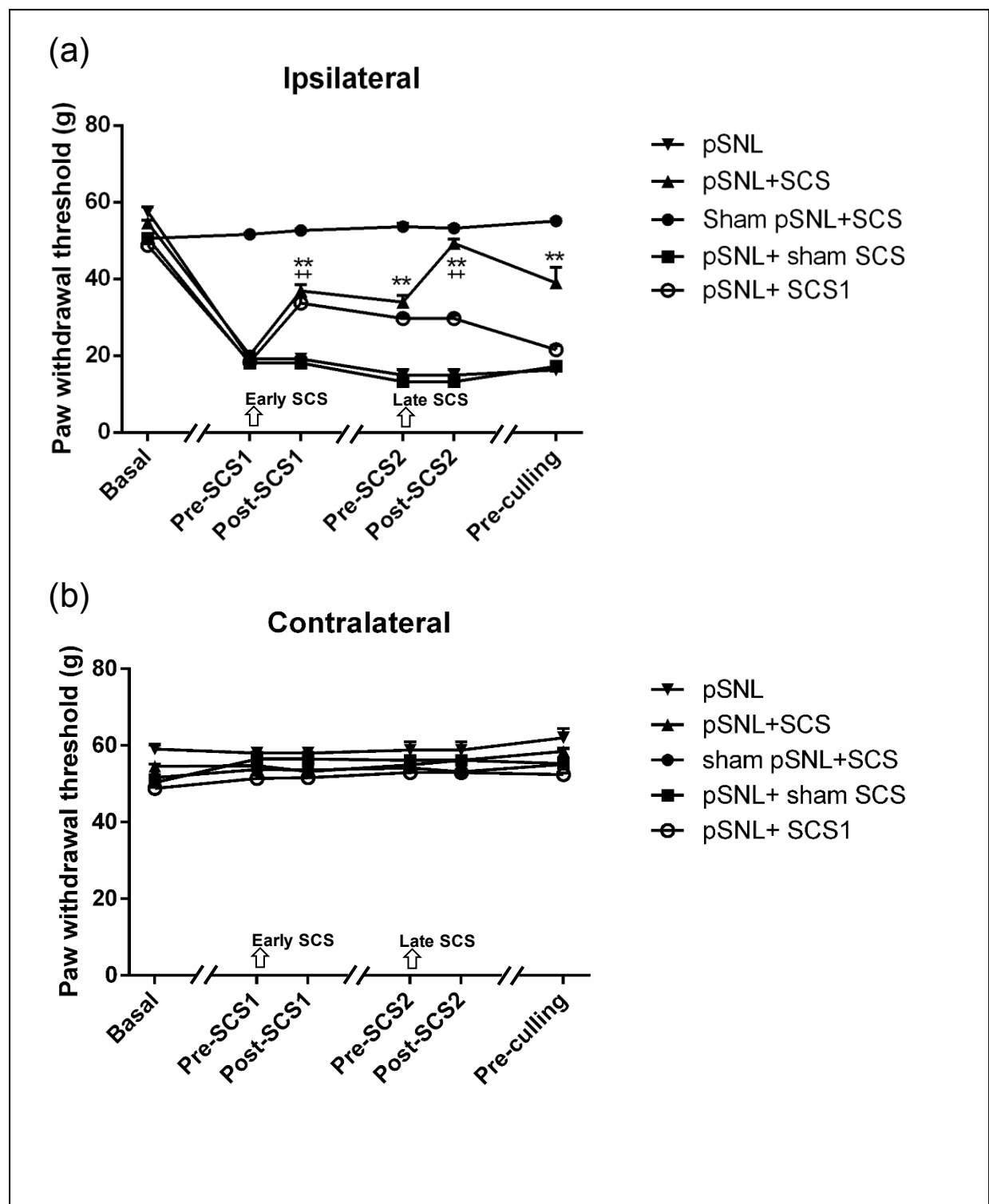


Fig. 2

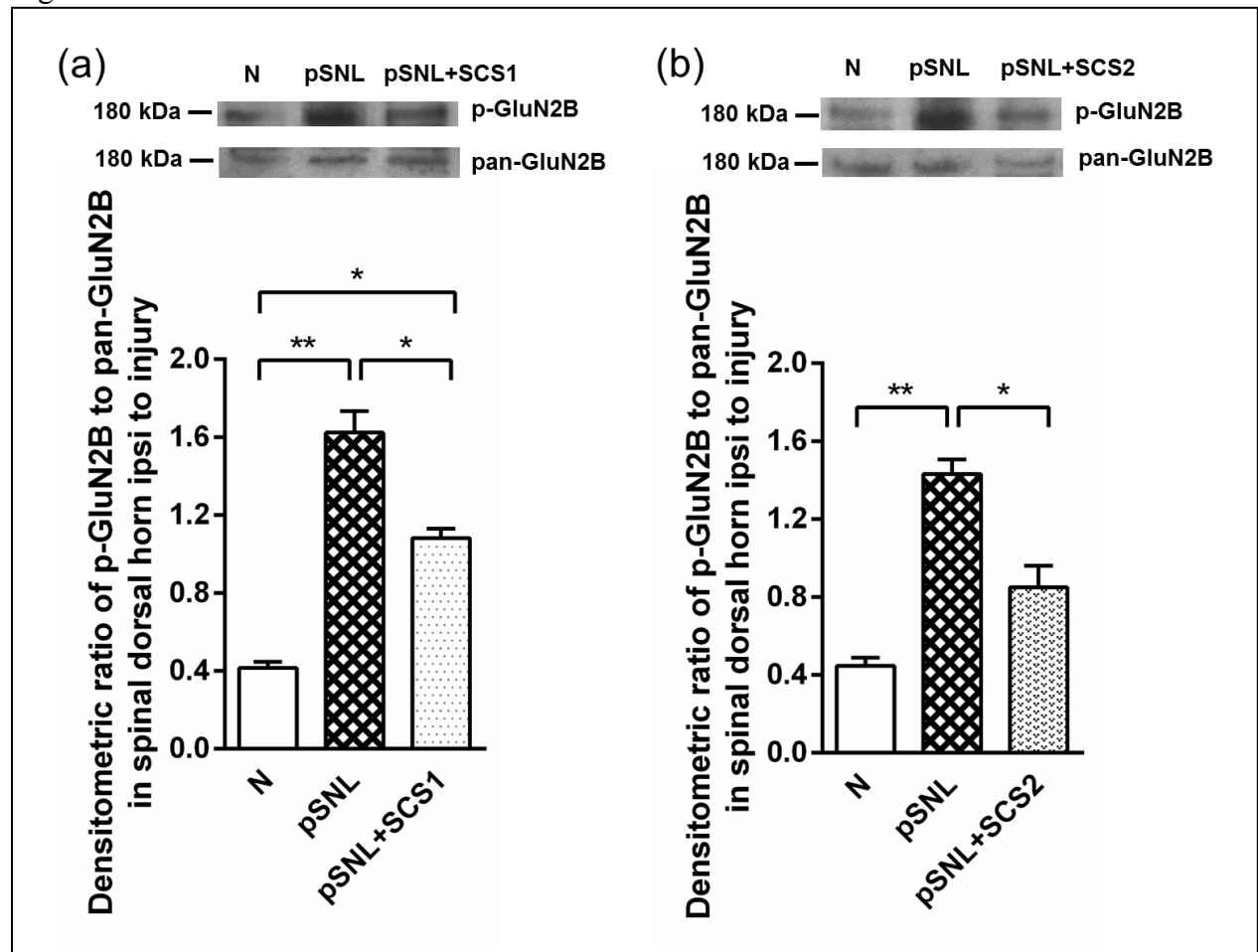


Fig. 3

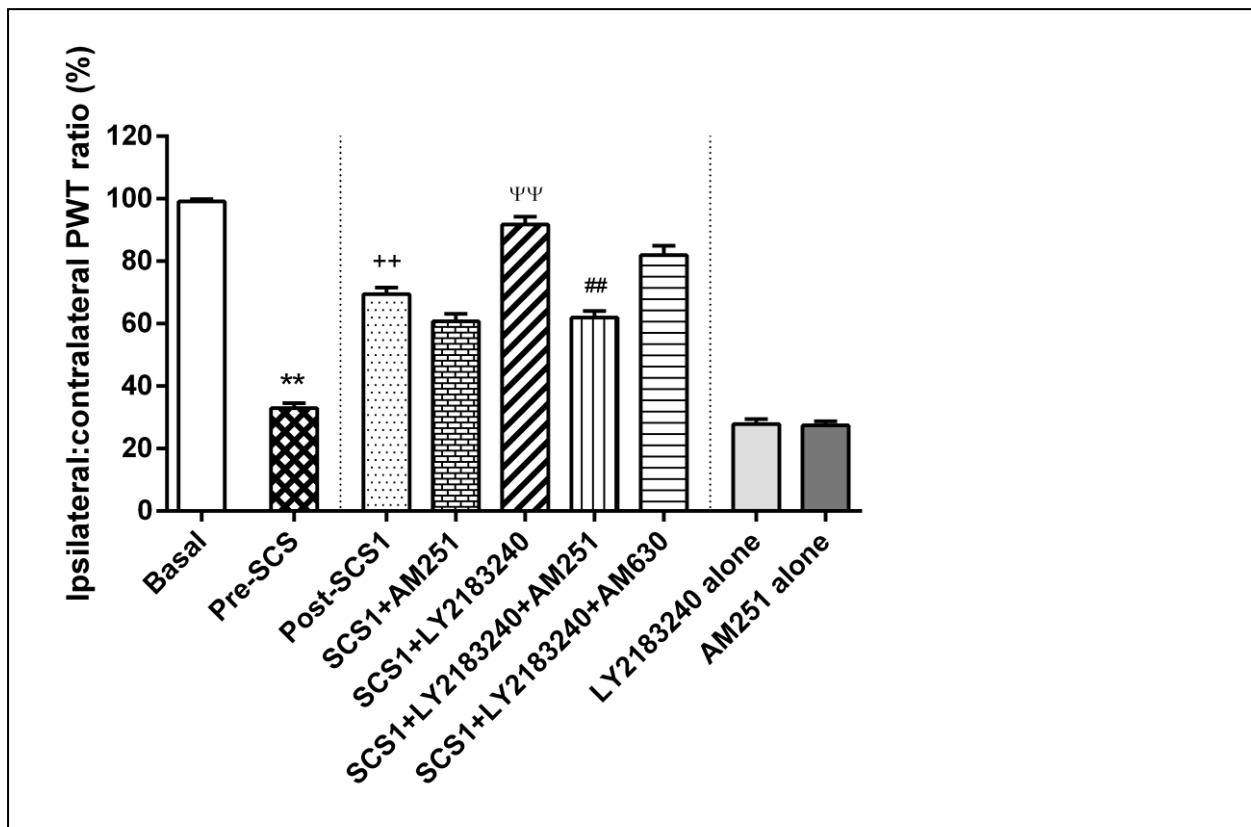


Fig. 4

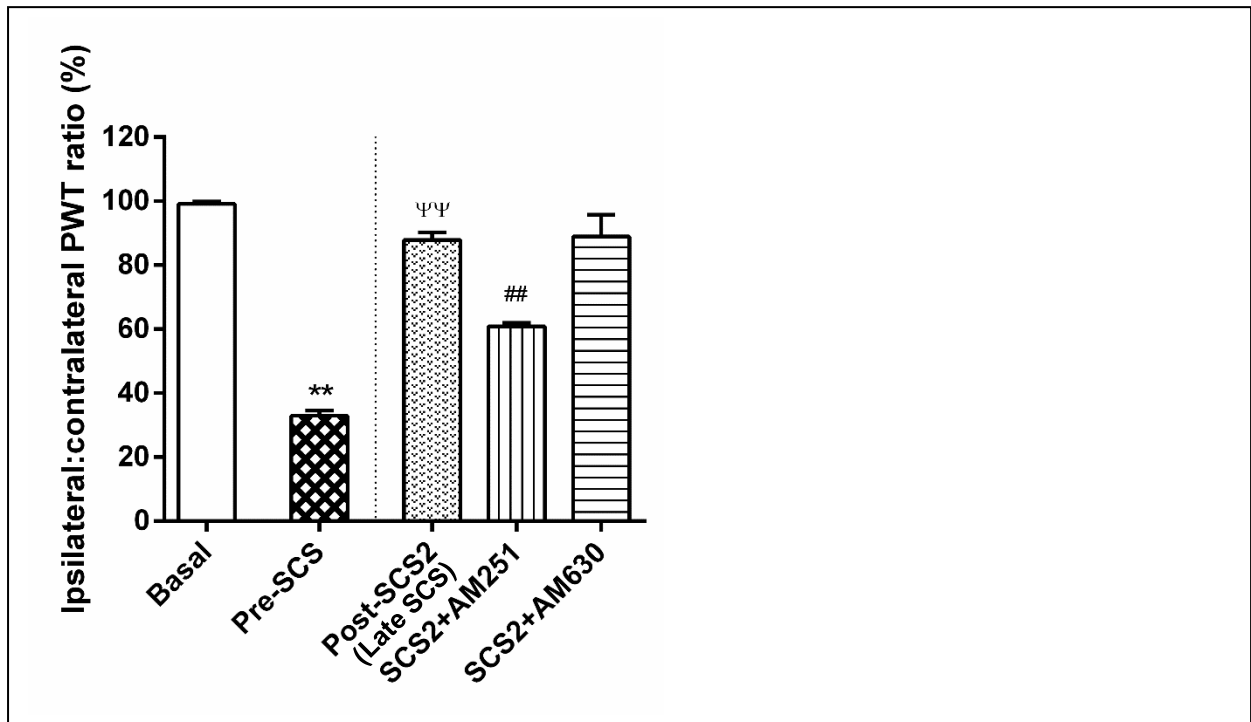


Fig. 5

